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DEVELOPMENT OF A QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION ASSAY SPECIFIC FOR *ORIENTIA TSUTSUGAMUSHI*

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Abstract. Two specific and sensitive polymerase chain reaction (PCR) assays were developed to detect and quantitate *Orientia tsutsugamushi*, the agent of scrub typhus, using a portion of the 47-kD outer membrane protein antigen/high temperature requirement A gene as the target. A selected 47-kD protein gene primer pair amplified a 118-basepair fragment from all 26 strains of *O. tsutsugamushi* evaluated, but it did not produce amplicons when 17 *Rickettsia* and 18 less-related bacterial nucleic acid extracts were tested. Similar agent specificity for the real-time PCR assay, which used the same primers and a 31-basepair fluorescent probe, was demonstrated. This sensitive and quantitative assay determination of the content of *O. tsutsugamushi* nucleic acid used a plasmid containing the entire 47-kD gene from the Kato strain as a standard. Enumeration of the copies of *O. tsutsugamushi* DNA extracted from infected tissues from mice and monkeys following experimental infection with *Orientia* showed 27–5,552 copies/μL of mouse blood, 14,448–86,012 copies/μL of mouse liver/spleen homogenate, and 3–21 copies/μL of monkey blood.

INTRODUCTION

Scrub typhus is a mild to fatal disease depending on both the *Orientia tsutsugamushi* strain encountered and the genetic background and physical condition of the patient. The disease typically presents with fever, headache, maculopapular rash, eschar (pathognomic lesion), lymphadenopathy, and central nervous system involvement.[1,2] Accurate diagnosis is important since without appropriate and effective antibiotic treatment, serious disease and high mortality rates (up to 50%) can occur.[1–5] Scrub typhus is caused by infection with *O. tsutsugamushi* following the bite of an infected trombiculid mite. The mite acts as both the reservoir and vector, but only the parasitic larval stage (chigger) feeds on humans and rodents. Geographic distribution of the disease occurs within an area of about 13 million km^2 including Afghanistan and Pakistan to the west; Russia to the north; Korea and Japan to the northeast; Indonesia, Papua New Guinea, and northern Australia to the south; and some smaller islands in the western Pacific.[1,5–7] Scrub typhus has been contracted in undisturbed rain forests, secondary vegetative growth areas, plantations, rice paddies, riverbanks, semiarid deserts, and urban areas.[2,7–10]

Historically, laboratory diagnosis of scrub typhus was most often performed by recognition of serum reactivity to *Proteus mirabilis* Kingsbury strain antigen (OXK) in the Weil-Felix test. Although the test is easy to perform and inexpensive, its lack of specificity and sensitivity (less than 50% of cases during the second week of illness are reactive) has led to the use of serologic assays that use *O. tsutsugamushi* antigens.[11,12] These assays include an indirect immunofluorescent antibody test,[13] an indirect immunoperoxidase assay,[12,14] an enzyme-linked immunosorbent assay (ELISA),[15] and a dot-blot immunoassay.[16] Recently, diagnostic assays using the immunodominant 56-kD outer membrane protein antigens[17–21] have eliminated the need to grow and purify *O. tsutsugamushi* in a biosafety level (BSL)-3 containment laboratory. Increased use of antigen-specific ELISAs and rapid flow through assays are expected due to an enhanced stability and consistency of the antigen preparation as well as a reduction in cost, transport, hazard, and reproducibility problems presently associated with whole-cell antigen preparations.[22] However, these serologic assays are still limited in clinical value because development of a detectable antibody response to *O. tsutsugamushi* usually occurs in the second week of illness,[23] there are no minimal titers established for diagnostic tests, and paired sera showing a rise in antibody titer are recommended for laboratory diagnosis of scrub typhus.[24]

Agent detection can be accomplished earlier after onset of disease than antibody detection and therefore decreases the time required for specific diagnosis. The most definitive means of agent detection is by culture of *O. tsutsugamushi* in laboratory animals, yolk sacs of embryonated chicken eggs, or tissue culture.[25] However, the procedures are slower and less efficient than serologic assays and require a sophisticated BSL-3 laboratory that most diagnostic laboratories do not have.

Due to the very low peak concentration of *O. tsutsugamushi* in patients' blood (approximately 5–10 organisms/μL), direct detection of the agent-specific antigens by antigen capture methods has been insensitive and requires preconcentration of the antigen.[26,27] However, with the advent of the polymerase chain reaction (PCR), where agent-specific nucleic acid can be amplified a million-fold, *O. tsutsugamushi* DNA can be readily detected with great sensitivity in clinical specimens such as blood and tissue samples,[28–30] as well as in arthropod vectors.[31,32] This sensitivity has been enhanced by the development of the nested PCR assays that are performed using two separate amplification steps.[32] In addition to detection, the amplicon can be used for restriction fragment length polymorphism (RFLP) typing or sequence analysis.[30–32]

Real-time quantitative PCR assays are as sensitive as nested PCR assays but have the additional advantages of 1) faster results, 2) potential for automation for high throughput, 3) multiplexing, and 4) quantitative information useful for clinical monitoring of appropriate response to treatment and prognosis, and for experimental studies. In this report, we describe the development of a real-time quantitative PCR (qPCR) procedure with an agent-specific fluorescent probe able to detect *O. tsutsugamushi* nucleic acid at a lower limit of detection of 3–10 target sequence copies/μL. Furthermore,

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we have demonstrated the successful application of this qPCR methodology by quantitating *O. tsutsugamushi* nucleic acid in cynomolgus monkey and Swiss CD-1 mouse tissue samples.

MATERIALS AND METHODS

Primers and probes. Sequences of primers and probe for *O. tsutsugamushi* were selected from the 47-kD outer membrane protein gene based on the sequences of the Karp, Kato, Gilliam, Boryong (L31934, L11697, L31933, and L319335 GenBank numbers, respectively), and TH1817 (Dasch GA, unpublished data) strains using Primer Express version 1.0 software (PE Applied Biosystems, Foster City, CA). The primer set (forward primer OtsuFP630: 5′-AAC TGA TTT TAT TCA AAC TAA TGC TGC T-3′ and degenerate reverse primer OtsuRP747: 5′-TAT GCC TGA GTA AGA TAC RTG AAT RGA ATT-3′) was capable of producing an amplicon of 118 basepairs. The 31-basepair fluorescent TaqMan probe OtsuPR665 (5′-6FAM-TGG GTA GCT TTG GTG GAC CGA TGT TTA ATC T-TAMRA-3′) was labeled at the 5′-end with 6-carboxyfluorescein (FAM) reporter dye and at the 3′-end with 6-carboxytetramethylrhodamine (TAMRA) quencher dye. The oligonucleotide primers were synthesized by Sigma Genosys (The Woodlands, TX), and the probe was synthesized at PE Applied Biosystems.

Sequences of primers and probe for the *Rickettsia*-specific qPCR assay were selected from a 17-kD gene consensus sequence derived from 21 species of *Rickettsia*. The degenerate forward primer R17K135F: 5′-ATG AAT AAA CAA GGK ACN GGH ACA C-3′, the degenerate reverse primer R17K249R: 5′-AAG TAA TGC RCC TAC ACC TAC TC-3′ and probe R17Kbprobe: 5′-6FAM-CGC GAC CCG AAT TGA GAA CCA AGT AAT GCG TCG CG-Black Hole Quencher (BHQ)-1-3′ were synthesized by Sigma Genosys. Degenerate positions contained equal molar base concentrations of adenine, guanine, cytosine, and thymine (N); guanine and thymine (K); adenine and guanine (R); and adenine, thymine, and cytosine (H). The presence of a target sequence of the small subunit ribosomal RNA (16S rRNA) gene was detected by a PCR assay using conserved eubacterial primers (forward: 5′-GTT CGG AAT TAC TGG GCG TA-3′ and reverse: 5′-AAT TAA ACC GCA TGC TCC AC-3′) as previously described.[33]

Nucleic acids. Three panels of nucleic acids used to assess the specificity of the PCR assays included 26 *O. tsutsugamushi* DNAs, 17 *Rickettsia* DNAs and 18 other bacterial DNAs (Table 1). The DNAs were extracted from the bacterial cultures[34–39] (Taye AB and others, unpublished data) by an automated extraction method and a nucleic acid extractor (Model 340A; Applied Biosystems) as previously described.[31] The bacteria were grown either in L929 cells (*O. tsutsugamushi*, *R. africae*, *R. sharonii*, *R. parkeri*, and *R. rhicpicephali*), Vero cells (other spotted fever group *Rickettsia* and *R. bellii*), P388D1 (*Neorickettsia*), and DH82 (*Ehrlichia*), chicken embryonated yolk sac (*R. prowazekii*, *R. typhi*, and *Francisella persica*), or on standard bacterial media (all other

TABLE 1
Orientia tsutsugamushi–specific sPCR and qPCR*

Orientia tsutsugamushi strains	47-kD sPCR	47-kD qPCR	*Rickettsia* isolates	47-kD sPCR	47-kD qPCR	17-kD qPCR	Other bacterial species	47-kD sPCR	47-kD qPCR	16S rRNA sPCR
Karp	+	+	*R. prowazekii* Breinl	–	–	+	*Ehrlichia chaffeensis*	ND	–	+
Kato	+	+	*R. typhi* Wilmington	–	–	+	*Neorickettsia sennetsu*	–	–	+
Gilliam	+	+	*R. typhi* Museibov	–	–	+	*N. risticii*	–	–	+
TA678 PP	+	+	*R. bellii* G2D	–	–	+	*Bartonella quintana*	–	–	+
TA686 PP	+	+	*R.* sp. 364-D	–	–	+	*B. vinsonii*	–	–	+
TA716 PP	+	+	*R. conorii* ITT	–	–	+	*Francisella persica*	ND	–	+
TA763 PP	+	+	*R. montana* OSU 85–930	–	–	+	*Legionella pneumophila*	–	–	+
TH1813	+	+	*R. africae* EthSFC84360	–	–	+	*L. bozemanii*	–	–	+
TH1814	+	+	*R. sharonii* ISTT CW	–	–	+	*L. micdadei*	–	–	+
TH1817	+	+	*R. parkeri* Maculatum C (CWPP)	–	–	+	*Proteus mirabilis*	–	–	+
TH1818	+	+	*R. slovaca* D	–	–	+	*Escherichia coli*	–	–	+
TH1819	+	+	*R. japonica* NT	–	–	+	*Citrobacter freundii*	–	–	+
TH1823	+	+	*R. sibirica* 3358	–	–	+	*Shigella flexneri*	–	–	+
AFC3	+	+	*R. rhipicephali* CWPP	ND	–	+	*Pseudomonas aeruginosa*	–	–	+
AFPL12	+	+	*R. honei* TT118	ND	–	+	*Vibrio cholerae*	–	–	+
AF245	+	+	*R. akari* Str #29	ND	–	+	*Aeromonas hydrophila*	–	–	+
AF312	+	+	*R. felis*	ND	–	+	*Staphylococcus aureus*	–	–	+
AF316	+	+					*Corynebacterium* sp.	–	–	+
AF338	+	+								
MAK110	ND	+								
MAK119	ND	+								
18-032460	ND	+								
MR32403	ND	+								
Garton	ND	+								
Brown	ND	+								
Domrow	ND	+								

* sPCR = standard polymerase chain reaction; qPCR = quantitative PCR; rRNA = ribosomal RNA; ND = not determined.

bacteria). Cat fleas, obtained from FleaData, Inc. (Freeville, NY), were constitutively infected with *R. felis* (> 95%)[40] and used to extract nucleic acid containing *R. felis* DNA with the DNeasy tissue kit (Qiagen, Valencia, CA). For the qPCR, the plasmid pWMC-Kt47, which contains the entire open reading frame of the 47-kD antigen gene of *O. tsutsugamushi* Kato strain in pVR1012 (Vical, San Diego, CA), was used in the amount of 10^7–10^0 copies/μL.

Swiss CD-1 mouse blood and tissue (liver/spleen) samples were collected 7 and 10 days after infection with *O. tsutsugamushi* Karp and Gilliam strains, respectively. The DNA was extracted from 150 μL of blood and 25 μL of homogenized liver/spleen samples using The Neasy tissue kit and eluted in 100 μL and 200 μL of the kit AE buffer (Qiagen), respectively.

Cynomolgus monkeys were infected with different amounts of *O. tsutsugamushi* Karp strain, bacteria and blood samples were collected every other day post-infection. The DNA was extracted from 100 μL of blood from each monkey using the DNeasy tissue kit and eluted in 50 μL of the kit AE buffer (Qiagen).

The maintenance and care of experimental animals complied with the Animal Welfare Act and the National Institutes of Health guidelines for the humane use of laboratory animals. The experiments reported herein were conducted according to the principles set forth in the "*Guide for the Care and Use of Laboratory Animals*," Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996.

Standard PCR (sPCR) and Real-time qPCR. The sPCR was conducted in 50-μL reaction volumes containing 1 μL of DNA template, 5 μL of 10× PCR buffer with 15 mM $MgCl_2$ (Perkin Elmer, Foster City, CA), 5 μL of 2 mM dNTPs (Idaho Technology, Salt Lake City, UT), 2.5 μL of each 10 μM primer, and 0.25 μL (5 units/μL) of AmpliTaq Gold DNA polymerase (Perkin Elmer). The reaction mixtures were incubated at 95°C for 10 minutes followed by 45 cycles of three-step amplification at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for seven minutes using a GeneAmp PCR system 9700 (Perkin Elmer). The PCR amplicons were visualized and compared to molecular weight standards (100-basepair ladder; Invitrogen, Carlsbad, CA) after electrophoresis on 2% agarose gels at 150 volts for approximately 40 minutes in a Horizon 58 gel electrophoresis system (Gibco-BRL Life Technologies, Inc., Gaithersburg, MD) and staining with ethidium bromide (Gibco-BRL Life Technologies, Inc.).

The real-time qPCRs for the *O. tsutsugamushi* 47-kD and the *Rickettsia* 17-kD gene assays were conducted in a total volume of 25 μL of qPCR mixture consisting of 1 μL of DNA template, 2.5 μL of 10× PCR buffer with 50 mM $MgCl_2$, 2.5 μL of 2 mM dNTPs (Idaho Technology), 0.25 μL (5 units/μL) of platinum Taq DNA polymerase (Invitrogen), 0.25 μL (10 μM) of each primer, and 0.5 μL (10 μM) of probe for the *O. tsutsugamushi* 47-kD assay or 0.75 μL (10 μM) of primer and 1.0 μL (10 μM) of probe for the *Rickettsia* 17-kD assay. Both qPCRs were incubated at 94°C for five minutes followed by 45–50 cycles of two-step amplification at 94°C for five seconds and 60°C for 30 seconds on a Cepheid (Sunnyvale, CA) SmartCycler System. Fluorescence was monitored during every thermal cycle at annealing step and data were analyzed with SmartCycler software (version 1.2b). Initial sensitivity and specificity determinations for the *O. tsutsugamushi* assay were conducted with the ABI Prism7700 Sequence Detection System (Applied Biosystems) and results were similar to those obtained with the Cepheid SmartCycler. Three no template controls were consistently negative for each reaction. Plasmid pWMC-Kt47 DNA (10^5 copies/μL) was used as a positive control and gave consistent threshold (Ct) values between 24 and 25 cycles for the *O. tsutsugamushi* 47-kD assay.

RESULTS

The *O. tsutsugamushi* 47-kD sPCR assay was first optimized with DNA extracted from the Karp strain of *O. tsutsugamushi*, and its specificity was evaluated with nucleic acid preparations from 19 strains of *O. tsutsugamushi*, 13 species of *Rickettsia* and 16 species of other bacteria (Table 1), and several eukaryotic cell DNAs (mouse, dog, flea, monkey, and chicken). The appropriate size amplicon (118 kb) was produced from all 19 strains of *O. tsutsugamushi*, but not from *Rickettsia* or other bacteria nucleic acid samples assessed, or the host DNAs in which the obligate intracellular bacteria were cultivated, indicating that the sPCR assay was specific for *O. tsutsugamushi*.

To decrease the time required to obtain a result and to accurately and sensitively quantitate the number of positive templates in the original samples, we developed a qPCR assay using a probe specific for a 31-basepair sequence within the *O. tsutsugamushi* 118-basepair amplicon. The qPCR assay detected all 26 strains of *O. tsutsugamushi* evaluated, but did not detect 17 *Rickettsia* or 18 other bacteria DNA preparations, or the host cell DNA extracted concurrently with the obligate intracellular bacteria (Table 1). The integrity of the *Rickettsia* and bacterial DNAs were confirmed with a qPCR assay based upon the conserved *Rickettsia* 17-kD antigen gene and a sPCR assay for the 16S rRNA gene (Table 1). The 26 strains of *O. tsutsugamushi* originated from seven different countries (Papua New Guinea, Japan, Burma, Thailand, China, Malaysia, and Australia) and were genetically quite diverse. Fourteen of the 26 strains were assessed in another study in which the 56-kD outer membrane protein gene sequences of 25 strains were determined and compared to each other and to 63 published strain sequences. The 14 strains from this study were well distributed with in the phylogenitc tree developed from the 88 total strains and had sequence homologies with the other 74 strains analyzed that ranged from a low of 70% to a high of 100% (Taye AB and others, unpublished data). In addition, Dasch and others determined that with the use of PCR-RFLP analysis, the19 strains included in this study also varied genetically among themselves and among 92 other strains evaluated.[36]

To determine the sensitivity of the real-time PCR assay for *O. tsutsugamushi*, we used plasmid pWMC-Kt47, which contains the open reading frame sequence of the Kato 47-kD gene that was ligated into the plasmid VR1012 (Vical). The concentration of the plasmid was determined by an optical density reading at 260 nm.[41] Serial ten-fold dilutions of pWMC-Kt47 in molecular biology–grade water (Sigma Chemical Company, St. Louis, MO) were performed, resulting in final target concentrations of 10^7–10^0 copies/μL. This assay consistently detected between 3 and 10 copies of the target sequence per reaction.

To evaluate the utility of the qPCR assay with laboratory-derived animal tissue samples, mouse and monkey blood and mouse liver/spleen homogenates infected with *O. tsutsugamushi* were tested. Blood collected from Swiss CD-1 outbred mice after inoculation with 1,000 50% murine lethal dose $(MuLD)_{50}$ produced 27–5,552 copies/μL of blood collected 7 and 10 days post-infection (Table 2). Homogenized liver/spleen samples from CD-1 mice inoculated with 1,000 $MuLD_{50}$ of either the Karp or Gilliam strain on days 7 and 10, respectively, were found to be positive (14,448–86,012 copies/μL) by the qPCR assay for the *O. tsutsugamushi* 47-kD gene (Table 2). Blood samples collected from six cynomolgus monkeys infected with 10^6–10^0 $MuLD_{50}$ produced 3–21 copies/μL of blood on days 8–18, depending upon the titer of the inoculum (Table 2). Enzyme-linked immunosorbent assays performed on these same blood samples indicated that *Orientia*-specific antibodies developed 4–8 days after detection of *Orientia*-specific DNA (Chattopadhyay S and others, unpublished data). These results are similar to those of earlier studies in which the presence of Orientiae in the blood of scrub typhus patients was shown to precede the humoral response of the host by approximately one week.[26,42]

DISCUSSION

The 47-kD gene sequences of *O. tsutsugamushi* strains Karp, Kato, Gilliam and Boryong are similar to those of the genes of the high-temperature requirement (HtrA) family of stress response proteins that have both chaperone and endoprotease activities (Chao C-C and others, unpublished data).[43] The HtrA gene is induced by different environmental stress conditions (e.g., elevated temperature) in a variety of bacteria (*Escherichia coli* originally as DegP, *R. prowazekii*, *R. typhi*, *R. conorii*, *Haemophilus influenzae*, *Brucella abortus*, *Yersinia entercolitca*, *Salmonella enterica*, etc.) and have been shown to contribute to the envelope protein management and potentially play a role in the pathogenesis for some of these species.[43] The HtrA family of proteins has also been found in eukaryotic organisms including humans ($hHtrA_1$, $hHtraA_2$, $hHtrA_3$, and $hHtrA_4$) where their activities appear to be more diverse, including roles in cell protein regulation, tumor suppression, and apoptosis.[44] Because of the immunogenicity and conservation of these genes and gene products, they have a potential role as reagents for diagnostic assays when target-specific domains can be identified.

In developing a PCR diagnostic assay for *O. tsutsugamushi*, primers were designed based upon sequences of this 47-kD antigen gene that were conserved among five strains of *O. tsutsugamushi* (Karp, Kato, Gilliam, Boryong, and TH1817), but not found in the HtrA gene of the closely related genus *Rickettsia* (GenBank Y11782 *R. prowazekii*, AE008583 *R. conorii*) or in the human homologs. The sequence for the 47-kD of *R. typhi* (HtrA homolog) currently listed in Genbank (D78346) and previously reported suggests a sequence very similar to that of *O. tsutsugamushi* 47-kD antigen gene,[43] but it is not found in the newly determined *R. typhi* genome sequence (Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX, http://hgsc.bcm.tmc.edu/microbial/Rtyphi). We did not observe a product produced by our primers in the *O. tsutsugamushi* 47-kD sPCR assay or a positive response in the *O. tsutsugamushi* 47-kD qPCR assay

TABLE 2
Real-time quantitative polymerase chain reaction (qPCR) detection and quantitation of *Orientia tsutsugamushi* in mouse and monkey tissues*

Host tissue	*O. tsutsugamushi* inoculum			Time (days) after inoculation until positive Ct	qPCR Ct cycle	Calculated copy number for extracted†/original sample‡	
	Strain	Individual	Dose ($MuLD_{50}$)				
Mouse blood	Karp	M1	10^3	7	32.37	935.8	623.9
		M2	10^3	7	32.39	926.3	617.5
		M3	10^3	7	30.15	3,287.7	2,191.8
		M4	10^3	7	38.86	40.0	26.7
		M5	10^3	7	32.43	905.1	603.4
		M6	10^3	7	29.50	8,327.6	5,551.7
		M7	10^3	7	32.44	902.7	601.8
	Gilliam	M1	10^3	10	34.30	419.1	279.4
		M2	10^3	10	31.84	1,616.3	1,077.5
		M3	10^3	10	Negative	0	0
		M4	10^3	10	37.87	54.6	36.4
		M5	10^3	10	34.80	307.9	205.3
Mouse liver/spleen	Karp		10^3	7	29.48	10,751.5	86,012
	Gilliam		10^3	10	31.94	1,806.0	14,448
Monkey blood	Karp		10^6	8	38.93	34.3	3.4
	Karp		10^4	14	37.11	101.2	10.1
	Karp		10^2	14	35.83	210.1	21.0
	Karp		10^1	18	36.16	173.2	17.3
	PBS		NA	NA	Negative	0	0

* $MuLD_{50}$ = murine lethal dose in which 50% of CD-1 Swiss outbred mice will die; Ct = number of cycles required to see specific fluorescence above the threshold of background fluorescence; this is considered a positive response; PBS = phosphate-buffered saline; NA = not applicable.

† Copy number is for 1μL of a total of 100 μL or 5 μl of a total of 50 μL of extracted DNA from mouse or monkey blood, respectively, and for 1 μL of a total of 200 μL of extracted mouse liver/spleen homogenate.

‡ Copy number is calculated for 1 μL of a total of 150 or 100 μL of blood from mouse or monkey, respectively, or for 1 μL of a total of 25 μL of liver/spleen homogenate. Therefore, copy number determined for 1 μL of blood is calculated by multiplying the copy number computed by the SmartCycler from the standard curve by 100 or 10 for mouse or monkey, respectively; that value is then divided by the volume of blood (150 or 100 μL for mouse or monkey, respectively); and for 1 μL of liver/spleen homogenate is calculated by multiplying the copy number computed by the SmartCycler by 200; that value is then divided by the volume of liver/spleen homogenate (25 μL). That is, mouse copy number from SmartCycler × 100/150 = copy number of *O. tsutsugamushi* 47-kD gene/μL of blood; mouse copy number from SmartCycler × 200/25 = copy number of *O. tsutsugamushi* 47-kD gene/μL of liver/spleen homogenate; monkey copy number from SmartCycler × 10/100 = copy number of *O. tsutsugamushi* 47-kD gene/μL of blood.

with two strains (Wilmington and Museibov) of *R. typhi* (Table 1). However, we did observe a positive reaction for these two *R. typhi* nucleic acid preparations with real-time PCR assays developed for the *Rickettsia* 17-kD antigen gene (Table 1) and the *R. typhi* outer membrane protein B gene (Flavin M and others, unpublished data). This suggests that what is currently listed as a sequence for *R. typhi* 47-kD in GenBank may be in error.

The presently described qPCR assay is clearly useful for the detection and enumeration of *O. tsutsugamushi* in research samples and laboratory animal specimens. Thus, the assay could be used to monitor scrub typhus vaccine and antibiotic efficacy in animal models. To date, this qPCR assay has been used successfully in our laboratory to evaluate the efficacy of the scrub typhus vaccine candidate, Kp r56, in mice and cynomolgus monkeys challenged by *O. tsutsugamushi* delivered by needle inoculation (Chattopadhyay S and others, unpublished data). With the development of a chigger challenge model for vaccine efficacy studies, this qPCR assay will be critical in the determination of the challenge dose of the *O. tsutsugamushi* provided by the infected chigger, a previously recognized fault associated with this method. Currently, the standard procedure for *O. tsutsugamushi* challenge of vaccinated laboratory animals is by the unnatural needle inoculation method, where knowledge of concentration of the dose is known. This needle challenge method could be supplanted by the chigger challenge procedure with the knowledge of the challenge dose of the chigger provided by the qPCR assay.

In addition, to the use of this assay with experimental animal specimens, it can also be used with human samples. Presently, this qPCR assay has been used to detect and enumerate *O. tsutsugamushi* Karp in infected human peripheral blood mononuclear cells (Rentas FJ and others, unpublished data). Therefore, this assay should be useful in diagnosing scrub typhus by detecting *O. tsutsugamushi* in patient samples (blood, eschar, and rash biopsies) shortly after symptoms appear. This early and rapid diagnosis would allow the initiation of agent-specific therapy, offering the best opportunity for quick and complete recovery of the patient.

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REFERENCES

1. Silpapojakul K, 1997. Scrub typhus in the western Pacific region. *Ann Acad Med Singapore 26:* 794–800.
2. Richards AL, Teik OC, Lockman JL, Rahardjo E, Wignall FS, 1997. Scrub typhus in an American living in Jakarta, Indonesia. *Infect Dis Clin Pract 6:* 268–273.
3. Yi KS, Chong Y, Covington SC, Donahue BJ, Rothen RL, Rodriguez J, Arthur JD, 1993. Scrub typhus in Korea: importance of early clinical diagnosis in this newly recognized endemic area. *Mil Med 158:* 269–273.
4. Smadel JE, Elisberg BL, 1965. Scrub typhus rickettsia. Horsfall FL, Tamm, I eds. *Viral and Rickettsial Infections of Man*. Fourth edition. Philadelphia: J.B. Lippincott, 1130–1143.
5. Rosenberg R, 1997. Drug resistant scrub typhus: paradigm and paradox. *Parasitol Today 13:* 131–132.
6. Kawamura A, Tanaka H, 1988. Rickettsiosis in Japan. *Jpn J Exp Med 58:* 169–184.
7. Traub R, Wisseman C, 1974. Ecology of chigger-borne rickettsioses. *J Med Entomol 3:* 237–303.
8. Traub R, 1954. Advances in our knowledge of military medical importance of mites and fleas due to postwar experiences in the Pacific area. *Recent Advances in Medicine and Surgery*. Washington, DC: Army Medical Service Graduate School, 284–294.
9. Strickman D, Tanskul P, Eamsila C, Kelly DJ, 1994. Prevalence of antibodies to rickettsiae in the human population of suburban Bangkok. *Am J Trop Med Hyg 51:* 149–153.
10. Tanskul P, Linthicum K, Watcharapichat P, Phulsuksombati D, Mungviriya S, Ratanatham S, Suwanabun N, Sattabongkot J, Watt G, 1998. A new ecology for scrub typhus associated with a focus of antibiotic resistance in rice farmers in Thailand. *J Med Entomol 35:* 551–555.
11. Brown GW, Robinson DM, Huxsoll DL, 1976. Scrub typhus: a common cause of illness in indigenous populations. *Trans R Soc Trop Med Hyg 70:* 444–448.
12. Tay ST, Kamalanathan M, Rohani MY, 2003. Detection of rickettsial antibodies using Weil-Felix (OXK and OX19) antigens and the indirect immunoperoxidase assay. *Southeast Asia J Trop Med Public Health 34:* 171–174.
13. Robinson DM, Brown G, Gan E, Huxsoll DL, 1976. Adaptation of a microimmunofluorescence test to the study of human *Rickettsia tsutsugamushi* antibody. *Am J Trop Med Hyg 25:* 900–905.
14. Yamamoto S, Minamishima Y, 1982. Serodiagnosis of tsutsugamushi fever (scrub typhus) by the indirect immunoperoxidase technique. *J Clin Microbiol 15:* 1128–1132.
15. Dasch GA, Halle S, Bourgeois AL, 1979. Sensitive microplate enzyme-linked immunosorbent assay for detection of antibodies against scrub typhus rickettsia, *Rickettsia tsutsugamushi. J Clin Microbiol 9:* 38–48.
16. Weddle JR, Chan TC, Thompson K, Paxton H, Kelly DJ, Dasch G, Strickman D, 1995. Effectiveness of a dot-blot immunoassay of anti-*Rickettsia tsutsugamushi* antibodies for serologic analysis of scrub typhus. *Am J Trop Med Hyg 53:* 43–46.

17. Kim IS, Seong SY, Woo SG, Choi MS, Chang WH, 1993. High-level expression of a 56-kilodalton protein gene (bor56) of *Rickettsia tsutsugamushi* Boryong and its application to enzyme-linked immunosorbent assays. *J Clin Microbiol 31:* 598–605.
18. Kim IS, Seong SY, Woo SG, Choi MS, Kang JS, Chang WH, 1993. Rapid diagnosis of scrub typhus by a passive hemagglutination assay using recombinant 56-kilodalton polypeptides. *J Clin Microbiol 31:* 2057–2060.
19. Ching WM, Wang H, Eamsila C, Kelly DJ, Dasch GA, 1998. Expression and refolding of truncated recombinant major outer membrane protein antigen (r56) of *Orientia tsutsugamushi* and its use in enzyme-linked immunosorbent assays. *Clin Diagn Lab Immunol 5:* 519–526.
20. Ching WM, Rowland D, Zhang Z, Bourgeois AL, Kelly D, Dasch GA, Devine PL, 2001. Early diagnosis of scrub typhus with a rapid flow assay using recombinant major outer membrane protein antigen (r56) of *Orientia tsutsugamushi. Clin Diagn Lab Immunol 8:* 409–414.
21. Wilkinson R, Rowland D, Ching W-M, 2003. Development of an improved rapid lateral flow assay for the detection of *Orientia tsutsugamushi* specific IgG/IgM antibodies. *Ann N Y Acad Sci 990:* 386–390.
22. Coleman RE, Sangkasuwan V, Suwanabun N, Ching W-M, Sattabongkot J, Eamsila C, Richards AL, Rowland D, Devine P, Lerdthusnee K, 2002. Comparative evaluation of selected diagnostic assays for the detection of IgG and IgM antibody to *Orientia tsutsugamushi* in Thailand. *Am J Trop Med Hyg 67:* 497–503.
23. Hechemy KE, Rikihisa Y, 2002. Immunoserology of the *Rickettsiales*, *Bartonella*, and *Coxiella*. Rose NR, Hamilton RG, Detrick B, eds. *Manual of Clinical Laboratory Immunology*. Sixth edition. Washington, DC: American Society for Microbiology Press, 528–541.
24. Watt G, Olson JG, 2000. Scrub typhus. Strickland GT, ed. *Hunter's Tropical Medicine and Emerging Infectious Diseases.* Eighth edition. Philadelphia, PA: W.B. Saunders Company, 443–445.
25. Ridgway RL, Oaks SC Jr, LaBarre DD, 1986. Laboratory animal models for human scrub typhus. *Lab Animal Sci 36:* 481–485.
26. Shirai A, Saunders JP, Dohany AL, Huxsoll DL, Groves MG, 1982. Transmission of scrub typhus to human volunteers by laboratory-reared chiggers. *Jpn J Med Sci Biol 35:* 9–16.
27. Kelly DJ, Richards AL, Temenak J, Strickman D, Dasch GA, 2002. The past and present threat of rickettsial diseases to military medicine and international public health. *Clin Infect Dis 34:* S145–S169.
28. Furuya Y, Yoshida Y, Katayama T, Kawamori F, Yamamoto S, Ohashi N, Tamura A, Kawamura A, 1991. Specific amplification of *Rickettsia tsutsugamushi* DNA from clinical specimens by polymerase chain reaction. *J Clin Microbiol 29:* 2628–2630.
29. Murai K, Tachibana N, Okayama A, Shishime E, Tsuda K, Oshikawa T, 1992. Sensitivity of polymerase chain reaction assay for *Rickettsia tsutsugamushi* in patients' blood samples. *Microbiol Immunol 36:* 1145–1153.
30. Horinouchi H, Murai K, Okayama A, Nagatomo Y, Tachibana N, Tsubouchi H, 1996. Genotypic identification of *Rickettsia tsutsugamushi* by restriction fragment length polymorphism analysis of DNA amplified by the polymerase chain reaction. *Am J Trop Med Hyg 54:* 647–651.
31. Kelly DJ, Dasch GA, Chan TC, Ho TM, 1994. Detection and characterization of *Rickettsia tsutsugamushi* (Rickettsiales: Rickettsiaceae) in infected *Leptotromibidium* (*Leptroptrombidium*) *fletcheri* chiggers (Acari: Trombiculidae) with the polymerase chain reaction. *J Med Entomol 31:* 691–699.
32. Ree H-I, Kim T-E, Lee I-Y, Jeon S-H, Hwang U-W, Chang W-H, 2001. Determination and geographic distribution of *Orientia tsutsugamushi* serotypes in Korea by nested polymerase chain reaction. *Am J Trop Med Hyg 65:* 528–534.
33. Ge H, Chuang Y-Y, Zhao S, Tong M, Tsai M-H, Temenak JJ, Richards AL, Ching W-M, 2004. Comparative genomics of *Rickettsial prowazekii* Madrid E and Breinl strains. *J Bacteriol 186:* 556–565.
34. Weisburg WG, Dobson ME, Samuel JE, Dasch GA, Mallavia LP, Baca O, Mandelco L, Sechrest JE, Weiss E, Woese CR, 1989. Phylogenetic diversity of the rickettsiae. *J Bacteriol 171:* 4202–4206.
35. Kelly DJ, Marana DP, Stover CK, Oaks EV, Carl M, 1990. Detection of *Rickettsia tsutsugamushi* by gene amplification using polymerase chain reaction techniques. *Ann N Y Acad Sci 590:* 564–571.
36. Dasch GA, Strickman D, Watt G, Eamsila C, 1996. Measuring genetic variability in *Orientia tsutsugamushi* by PCR/RFLP analysis: a new approach to questions about its epidemiology, evolution, and ecology. Kazar J ed. *Rickettsiae and Rickettsial Diseases*. Vth International Symposium. Bratislava, Slovakia: Slovak Academy of Sciences, 79–84.
37. Dasch GA, Jackson LM, 1998. Genetic analysis of isolates of the spotted fever group of rickettsiae belonging to the *R. conorii* complex. *Ann N Y Acad Sci 849:* 11–20.
38. Eremeeva ME, Ching W-M, Wu Y, Silverman DJ, Dasch GA. Western blotting analysis of heat shock proteins of *Rickettsiales* and other eubacteria. *FEMS Microbiol Lett 167:* 229–237.
39. Chan TC, Jiang J, Temenak JJ, Richards AL, 2003. Development of a rapid method for determining the infectious dose $(ID)_{50}$ of *Orientia tsutsugamushi* in a scrub typhus mouse model for the evaluation of vaccine candidates. *Vaccine 21:* 4550–4554.
40. Boostrom A, Beier MS, Macaluso JA, Macaluso KR, Sprenger D, Hayes J, Radulovic S, Azad AF, 2002. Geographic association of *Rickettsia felis*-infected opossums with human murine typhus, Texas. *Emerg Infect Dis 8:* 549–554.
41. Davis LG, Dibner MD, Battey JF, 1986. Optical density analytical measurements. Basic Methods in Molecular Biology. New York: Elsevier Science Publishing Co. Inc., 327–328.
42. Smadel JE, Ley HL Jr, Diercks FH, Traub R, 1950. Immunity in scrub typhus: resistance to induced reinfection. *Arch Pathol 50:* 847–861.
43. Kim J-H, Hahn M-J, 2000. Cloning and sequencing of the gene encoding the candidate HtrA of *Rickettsia typhi. Microbial Immunol 44:* 275–278.
44. Clausen T, Southan C, Ehrmann M, 2002. The HtrA family of proteases: implications for protein composition and cell fate. *Mol Cell 10:* 443–455.